IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant: Peter M. Glazer and Pamela A. Havre

Serial No.: 09/783,338 Group Art Unit: 1637

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For: *CHEMICALLY MODIFIED OLIGONUCLEOTIDE FOR SITE-DIRECTED MUTAGENESIS*

Assistant Commissioner for Patents
Washington, D.C. 20231

DECLARATION UNDER 37 C.F.R. § 1.132
OF DR. PETER M. GLAZER

Sir:

I, Peter M. Glazer, hereby declare that:

1. I am an inventor of the above-identified application.

2. I am a Professor in the Departments of Therapeutic Radiology and Genetics at Yale University School of Medicine, New Haven, Connecticut.

3. I have reviewed the Decision by the Board of Patent Appeals and Interferences in connection with the parent (U.S.S.N. 08/083,088) of the above-identified application (Appeal No. 1997-2520; heard January 25, 2001).

4. I understand that claims 6-14 of parent application U.S.S.N. 08/083,088 have been rejected under 35 U.S.C. § 112, first paragraph, on the basis that the specification does not enable those of skill in the art to use the claimed method for targeted mutagenesis *in vivo*. The

following experiments demonstrate that the claimed methods are enabled for one of ordinary skill in the art as of the time we filed the parent application, June 25, 1993.

5. One of ordinary skill in the art as of 1993, would have been a post-doctoral researcher with experience in design and synthesis of oligonucleotides, methods for transformation of cells and animal tissues, and assays for mutagenesis of target molecules.

6. Targeted Mutagenesis *In Vivo*

A study was performed to show the use of triple helix-forming oligonucleotides (TFOs) to mediate targeted genome modification as an alternative strategy for genetic manipulation. Oligonucleotides can bind to DNA in the major groove in a sequence-specific manner to form triple helices. See Chan, et al., *"Triplex DNA: fundamentals, advances, and potential applications for gene therapy"*, J. Mol. Med. 75: 267-82, 1997. Triplex formation is favored at polypurine/polypyrimidine regions of DNA and can occur with the third strand oriented either parallel or anti-parallel to the purine strand of the duplex. In the parallel motif, the canonical triplets are T.A:T and C.G:C.; whereas in the anti-parallel motif, the triplets are A.A:T and G.G.:C.

Based on this sequence-specific binding and potential for molecular recognition of unique genomic sites, a strategy in which the third strand was conjugated to a mutagen, such as psoralen, was initially designed so that the sequence specificity of the third strand binding could be conferred on the action of the mutagen. See Havre, et al., "*Targeted mutagenesis of DNA using triple helix-forming oligonucleotides linked to psoralen*", Proc. Natl. Acad. Sci. USA. 90: 7879-83, 1993; Havre, "*Targeted mutagenesis of simian virus 40 DNA mediated by a triple helix-forming oligonucleotide*," J. Virology. 67: 7324-31, 1993.

This approach showed that psoralen-conjugated TFOs transfected into monkey COS cells can induce base pair-specific mutations within the supF mutation reporter gene in a simian virus 40 (SV40) genome in the cells, at frequencies in the range of 1%-5%. See Wang, G., et al., *"Targeted mutagenesis in mammalian cells mediated by intracellular triple helix formation,"* Mol. Cell. Biol. 15: 1759-1768, 1995. The key finding in this work was that the binding affinity of the TFO to its target site, as measured *in vitro*, was highly correlated with its intracellular activity. TFOs with Kd's (equilibrium dissociation constant) in the range of 10^{-9} M were active; those with Kd's of 10^{-6} M were not.

7. This work was recently extended to psoralen-TFO-mediated knock out of chromosomal genes. In one study, the supF reporter integrated into the chromosome of mouse fibroblasts was used as a target. See Vasquez, et. al., *"Chromosomal mutations induced by triplex-forming oligonucleotides in mammalian cells"*, Nucleic Acids Res. 27: 1176-81, 1999. Again, only high-affinity TFOs were active, achieving targeted mutagenesis frequencies of 0.1%. In the supF experiments, essentially unmodified G-rich oligonucleotides (except for 3′ end capping) designed to bind in the anti-parallel motif were used. A set of experiments to target the HPRT gene in hamster CHO fibroblasts (see Majumdar, et al., *"Targeted gene knockout mediated by triple helix forming oligonucleotides"*, Nature Genet. 20: 212-214, 1998), in contrast, used a series of T-rich psoralen TFOs (because the A-rich target favored the parallel motif). In these experiments, the unmodified TFOs were ineffective. A second conjugation of the TFO to an intercalator, either acridine or pyrene, was needed to provide additional binding affinity. Such doubly modified TFOS yielded HPRT mutagenesis at frequencies in the range of 10^{-4} to 10^{-3},

following electroporation of the TFOs into the cells, coupled with UVA irradiation to activate the psoralen.

8. However, the mutagenesis induced in the supF and HPRT chromosomal targets, while site specific in the sense that all the mutations clustered around the third strand binding site, was somewhat variable. Hence, these experiments suggested that psoralen-coupled TFOs may be useful for gene-specific knock out but not necessarily for predictable base pair-specific mutagenesis. As a research tool, however, psoralen TFOs have served as useful reagents to prove the ability of oligonucleotides to bind as third strands to chromosomal sites in living cells. The importance of this result is to establish the concept that DNA binding molecules can be used to direct site-specific genome modification and to show that the cell and nuclear membranes and the packaging of the DNA into chromatin are not absolute barriers to gene targeting with antigene oligonucleotides.

9. In the course of this work with psoralen-TFOs, it was observed that unconjugated TFOs were also capable of inducing mutations in the target gene, at least when the binding affinity was sufficiently high. See Wang, et al., *"Mutagenesis in mammalian cells induced by triple helix formation and transcription-coupled repair,"* Science 271: 802-805 (1996). This effect was shown to be a consequence of the stimulation of DNA repair by the formation of the triple helix, which seems to be recognized by the nucleotide excision repair complex as a "lesion."

10. Based on the concept that third strand binding, with or without psoralen coupling, can trigger DNA repair, we hypothesized that such binding might also be recombinogenic due to the production of repair-dependent DNA strand breaks. This work was reported by Faruq, et al.

"*Triple-helix Formation Induces Recombination in Mammalian Cells via a Nucleotide Excision Repair-Dependent Pathway*" Mol. Cell. Biol. 20(3):990-1000 (February 2000).

Using an SV40 vector containing two mutant copies of the supF gene, we found that both psoralen TFOs (see Faruqi, et al., "*Recombination induced by triple helix-targeted DNA damage in mammalian cells*", Mol. Cell. Biol. 16: 6820-6828, (1996)) and plain TFOs (unpublished data) can trigger recombination within an SV40 virus genome. This induced recombination in the case of the TFO without psoralen was found to be dependent on the presence of functional XPA protein, the key recognition factor in NER, a result consistent with our hypothesis.

These results were extended to a chromosomal target, in which two mutant thymidine kinase (TK) genes were integrated into a single chromosomal site in mouse fibroblasts. Transfection (via cationic liposomes) of the cells with high-affinity TFOs targeting a region in between the two TK genes yielded recombination at a frequency of approximately 10^{-4}, about 100-fold above background (unpublished data). When the TFOs were micro-injected into the nuclei of the cells (2000 copies/cell), the yield of recombinants increased to 1%-2%, 10,000-fold over background. Analysis of the recombinant clones revealed all the recombination events involved gene conversion rather than cross-over recombination.

Our observation of the ability of third strand binding to provoke DNA repair and stimulate recombination led us to develop a strategy to mediate targeted gene conversion using a TFO linked to a short DNA fragment homologous to the target site (except for the base pair to be corrected). See Chan, et al., "*Targeted correction of an episomal gene in mammalian cells by a short DNA fragment tethered to a triplex-forming oligonucleotide*", J Biol Chem. *274:* 11541-8, 1999. In this bi-functional molecule, the TFO domain mediates site-specific binding to target the

molecule to the desired gene. This binding also triggers repair to sensitize the target site to recombination. The tethered homologous donor fragment can participate in recombination and/or gene conversion with the target gene to correct or alter the nucleotide sequence.

Using a bi-functional oligomer with a 40-mer donor domain and a 30-mer TFO domain, correction of a single base pair mutation in the supF reporter gene within an SV40 vector in COS cells was achieved. Correction frequencies were in the range of 0.1% with the full bi-functional molecule. Oligomers consisting of either domain above or of either domain substituted with heterologous sequences reduced activity by 10-fold or more. The donor domain alone consistently did mediate some gene correction, as would be expected, based on the known ability of DNA fragments to mediate some level of recombination. However, there was a clear synergism due to combination with the TFO domain.

11. In summary, our work suggests that high-affinity DNA binding ligands, such as TFOs, can be used to mediate site-specific genome modification. This capacity derives not only from the ability of TFOs to bind as third strands with sequence specificity but also from the ability of the resulting triple helices to provoke repair and recombination, leading to directed mutagenesis, recombination, and potentially gene correction.

12. Specific Mutations Induced by Triplex-Forming Oligonucleotides in Mice.

Triplex-forming oligonucleotides (TFOs) recognize and bind to specific duplex DNA sequences and have been used extensively to modify gene function in cells.

Although germ line mutations can be incorporated by means of embryonic stem cell technology, little progress has been made toward introducing mutations in somatic cells of living organisms. Here we demonstrate that TFOs can induce mutations at specific genomic sites in

somatic cells of adult mice. Mutation detection was facilitated by the use of transgenic mice bearing chromosomal copies of the *supF* and *cII* reporter genes. Mice treated with a *supF*-targeted TFO displayed about fivefold greater mutation frequencies in the *supF* gene compared with mice treated with a scrambled sequence control oligomer. No mutagenesis was detected in the control gene (*cII*) with either oligonucleotide. These results demonstrate that site-specific, TFO-directed genome modification can be accomplished in intact animals. By inducing site-specific mutations in the genome, heritable changes can be achieved in gene function and expression.

By inducing site-specific mutations in the genome, heritable changes can be achieved in gene function and expression. Transgenic mice (C57BL/6 mice containing multiple copies of a chromosomally integrated λsupFG1 vector, designated 3340) were generated as described by Narayanan, et al., *Proc. Natl. Acad. Sci. USA* 94:3122 (1997) containing a 30-base pair (bp) triplex-targeted site within the *supFG1* mutation-reporter gene. We previously demonstrated TFO-targeted mutations in the *supFG1* gene *in vitro* on intracellular plasmid targets and on a chromosomal locus in a fibroblast cell line established from these mice (Wang, et al., *Mol. Cell. Biol.* 15, 1759 (1995); Wang, et al., *Science* 271, 802 (1996); and Vasquez, et al., *Nucleic Acids Res.* 27, 1176 (1999)). The results revealed 10- to 100-fold induction of site-specific mutations in cells treated with the specific TFO. Although psoralen conjugation to the TFO, coupled with UVA (ultraviolet, long wave) irradiation, generally increased the frequency of mutations, targeted mutagenesis was seen even without psoralen photoproduct generation, suggesting a substantial triplex-mediated process of mutagenesis. On the basis of these results, the oligonucleotides in this work were not conjugated to psoralen, and no other DNA-damaging

agent (or mutagen) was required to induce mutagenesis. This affords an advantage in that UVA activation is not required, and there is no apparent toxicity to the animals.

Mice were given daily intraperitoneal (i.p.) injections with 1 mg day $^{-1}$ of either AG30 or SCR30 for five consecutive days. AG30 is the specific TFO designed to bind to the polypurine site in the *supFG1* gene, whereas SCR30 is a control oligonucleotide with the same base composition as AG30 but with a scrambled sequence that differs at 12 positions. AG30 (5'AGGAAGGGGGGGGTGGGGGAGGGGGg-3') binds with high affinity to the 30-bp polypurine target site in the *supFG1* reporter gene. SCR39 (5'GGAGGAGTGGAGGGGAGTGAGGGGGGGGGG-3') is a control oligonucleotide with the same base composition as AG30, but a scrambled sequence (differing from AG30 at 12 positions). All oligonucleotides were synthesized with a 3' propanolamine group to prevent degradation. Using gel mobility shift assays and deoxyribonuclease I (Dnase I) footprinting, we found that AG30 binds with high affinity (equilibrium dissociation constant ~10^{-9} M) and specificity to the target site, whereas SCR30, having mismatches in the triplex binding code, does not. Then days after injection, mouse tissues were collected for mutation analysis. Transgenic mice (3340) were generated with about 15 copies of the λsupFG1 shuttle vector, containing the *supFG1* tRNA suppressor gene, integrated on chromosome 7 (Narayanan, et al., (1997)). Mice (8 weeks of age) were injected (i.p.) with oligonucleotides for 5 days, and 10 days after the last injection, mice were killed and tissue collected. Genomic DNA was isolated from mouse tissues, and λ packaging extracts were used to excise and package the vector DNA into viable phage particles for analysis in a lacZ(am) strain of *Excherichia coli* to detect mutations that occurred in the mouse (Narayanan, et al., (1997)). In the presence of a wild-type *supFG1*

gene, the amber mutation in the β-D-thiogalactopyranoside) and X-gal, whereas the mutant plaques are white. The combined mutation frequencies in a variety of tissues from AG30-treated mice were increased by about fivefold compared with tissues from SCR30-treated mice (Table 1). The mean mutation frequencies in liver, skin, kidney, colon, small intestine, and lung from AG30-treated mice were significantly higher than those from SCR30-treated mice (*P* values calculated by the Student's *t* test are listed in Table 1). These data demonstrate an oligonucleotide-specific induction of mutagenesis in mice. Additionally, these data indicate efficient tissue uptake and distribution of oligonucleotides in mice after i.p. injections.

In a previous study, the tissue uptake and distribution after i.p. administration of G-rich, propylamine end-capped, phosphodiester oligonucleotides (similar to AG30 and SCR30) was examined (Zendegui, et al. Nucleic Acids Res. 20, 307 (1992)). This study demonstrated substantial uptake in a number of tissues except for the brain. Because oligonucleotides do not efficiently cross the blood-brain barrier after i.p. injection, mutagenesis in brain tissue was measured as an internal control. Consistent with this, mutagenesis was not induced above background levels in brain tissues analyzed from mice treated with either AG30 or SCR30(Fig. 1A). In contrast, all other tissues tested from AG30-treated animals showed an average five-fold increased induction in mutagenesis compared with tissues from SCR30-treated animals (Fig. 1A).

The levels of mutagenesis obtained from mice injected with SCR30 were similar to those observed in mice injected with phosphate-buffered saline (PBS) only (Fig. 1A), showing that SCR30, even at a dose of 1 mg day^{-1} for 5 days, does not have any detectable mutagenic effect in the animals. The values for both the SCR30- and PBS-injected animals, furthermore, were

essentially the same as those observed in untreated animals in the same 3340 lineage (9) (Fig. 1A). These results not only provide additional evidence that AG30-mediated mutation induction is occurring through a sequence-specific, triplex-mediated mechanism, but also indicate that nonspecific oligonucleotides are not generally mutagenic in animals.

To further confirm that the induced mutagenesis obtained from AG30 treatment resulted from a triple-helix-dependent event and not from some hypothetical, nonspecific effect of AG30 on DNA metabolism, we tested the effect of AG30 and SCR30 in the Mutamouse mutagenesis model (Myhr, Environ. Mol. Mutagen, 18, 308(1991)). We chose to use the lambda *cII* gene as a mutation reporter, because this locus has been well studied (Jakubczak, et al., Proc. Natl. Acad. Sci. USA 93, 9073 (1996)) and does not contain the AG30 triplex target site. The *cII* control gene showed no induction of mutagenesis in animals treated with either AG30 or SCR30 compared with background levels (Table 2). These results rule out any nonspecific mutagenic effect of AG30 and are consistent with a gene-specific, triplex-mediated effect of AG30 in inducing an increased level of mutagenesis in the *supFG1* gene of 3340 mice.

Mutant plaques were isolated from a variety of tissues from AG30-treated animals, and the *supFG1* genes were analyzed by DNA sequencing. The sequences of *supFG1* mutations from a number of AG30-treated mouse tissues are listed in Fig.1B. The results are consistent with a TFO-directed effect with the majority (85%) of the mutations concentrated with the 30-bp triplex target site. Nearly 40% of the mutations are single-base insertions or deletions in a stretch of eight contiguous Gs. It is possible that these mutations resulted from slippage errors during TFO-induced repair, because this site has been shown previously to be prone to slippage events (Narayanan, et al., 1997; Vasquez, et al., 1999).

Indeed, the genome sites conducive to high-affinity triple-helix formation (consisting of polypurine sequences with segments of mononucleotide repeats) are precisely the kinds of sequences that are hot spots for insertion and deletion mutagenesis in a template dislocation and slippage mechanism, as proposed by Streisinger, et al., Cold Spring harbor Symp. Quant. Biol. 31, 77 (1966). As such, the 30-bp polypurine triplex target site in *supFG1* is an "at-risk motif" (Gordenin, et al., Mutat. Res. 400, 45 (1998)) that is a hot spot for both spontaneous and induced mutagenesis. Even in control animals, the 30-bp, G-rich polypurine site is a hot spot for mutations, with about 50% of spontaneous mutations occurring in that site, and even more in the absence of DNA mismatch repair (Narayanan, et al., (1997)). However, specific TFO treatment increases the proportion of mutations at this site and also increases the absolute mutation frequency at this site more than fivefold, in a highly sequence-specific manner. Hence, the hypermutability of G-rich (or A-rich) sites is a property that actually enhances the utility of TFOs that preferentially bind to such sites in efforts directed at somatic gene knockout.

In a previous study of directed mutagenesis by psoralen-conjugated TFOs within the *hprt* gene in Chinese hamster ovary cells (Majumdar, et al., Nature Genet. 20, 212 (1998)), mostly large deletions rather than single-base insertions or deletions were seen. However, in that study, the polypurine target site was situated within an intron, and so the type of slippage mutations we observed would have had no effect on the coding region and so would not have been detectable. In another study where psoralen-conjugated TFOs were used to target mutations in yeast, only single-base substitution mutations within a specific ochre codon were seen, rather than slippage events (Barre, et al., *Proc. Natl. Acad. Sci. USA* 97, 3084 (2000)). However, the yeast study made use of an assay requiring specific reversion of the ochre stop codon, instead of a forward

mutation assay, and so neither slippage mutations nor base substitutions elsewhere in the target site would have been observable.

We have investigated the potential of a triplex-based strategy to target mutations to a specific gene in the somatic tissues of mice. The results demonstrate substantial induction of mutagenesis in a variety of tissues with a TFO (AG30) designed to bind to the chromosomal triplex target site in transgenic animals. We detected no induction of mutagenesis with either AG30 or SCR30 in the *supFG1* gene in brain tissue, or in the *cII* control gene lacking the AG30 triplex target site. Additionally, no induced mutagenesis was detected in the target gene in any of the tissues tested with the control oligonucleotide, SCR30. These findings demonstrate the successful application of a site-specific, DNA-binding reagent to specifically induce genome modifications on a chromosomal target in intact animals.

Although the mechanism of the triplex-directed mutagenesis is not fully established, evidence suggests that the triplex structure can invoke the nucleotide excision repair machinery, either directly or by transcription-coupled repair (Hanawalt, Science 266, 1957 (1994)). This possibility is consistent with results of previous studies that demonstrate a lack of triplex-induced mutagenesis or recombination in nucleotide excision repair-deficient cells (Wang, et al., Mol. Cell. Biol. 15, 1759 (1995), Faruqi, et al., Mol. Cell. Biol. 20, 990 (2000)). The single-base insertions and deletions that were detected in the triplex target site in mice may therefore be a result of template misalignment in association with TFO-induced repair of this "at risk" site (Gordenin, et al., 1998).

The ability to induce mutations in living animals by means of TFOs affords a powerful strategy to modify the genome. In addition, recent developments in oligonucleotide technology

have advanced the potential of gene medicines for other approaches, such as specifically binding to RNA [antisense and ribozymes] (Galderisi, et al., J. Cell Physiol. 181, 251 (1999); Bramlage, et al., Trends Biotechnol. 16, 434 (1998)), DNA [anti-gene] TFOs, peptide nucleic acids, polyamides, and RNA/DNA chimeras (Vasquez, et al., Trends Biochem. Sci. 23, 4 (1998), Good, et al., Antisense Nucleic Acid Drug Dev. 7, 431 (1997); Gottesfeld, et al., Nature 387, 202 (1997); Cole-Strauss, et al., 273, 1386 (1996)), and protein [aptamer oligomers] (Famulok, Curr. Opin. Struct. Biol. 9, 324 (1999)) targets. Our data showing a lack of mutagenesis by the scrambled sequence control (SCR30) are promising for these other nucleic acid-based strategies because nonspecific oligonucleotides do not appear to be mutagenic.

In addition, for gene-targeting technologies to provide a therapeutic benefit, oligonucleotides must be shown to be capable of binding specifically to chromosomal targets in intact animals. The *in vivo* distribution of oligonucleotides has been studied with a variety of modified oligonucleotides in mice (Zendegui, et al., (1992); Chen, et al., Drug Metab. Dispos. 18, 815 (1990); Agrawal, et al., Proc. Natl. Acad. Sci. USA 88, 7595 (1991)). These studies established that small DNA molecules can be administered by i.p. or intravenous injections and gain access to tissues (outside the central nervous system) and to cell nuclei. The work presented here extends these studies by demonstrating that chromosomal DNA throughout the somatic tissues of an animal can be targeted by nucleic acids. This finding has important implications for the ability to use small molecules to modify gene structure and function in animals.

Although the overall efficiency of mutagenesis demonstrated with AG30 is low, our data provide evidence that sequence-specific, DNA-modifying reagents are useful in intact animals.

Table 1. Targeted mutagenesis of the *supFG1* gene in 3340 mice by TFOs. Mice were injected i.p. with 1 mg of TFO per day for 5 days (~50 mg kg^{-1} day^{-1}). AG30 is the specific TFO, SCR30 is the control TFO. Values of mutation frequency for skin, liver, and kidney are reported as the mean ± the difference between the two values. *P* values were calculated by the Student's *t* test.

TFO	Tissue	No. of mice	Mutants/total plaques	Mutation frequency ($x10^{-5}$)	*P* value
AG30	Skin	5	32/144,768	21 ± 10	
SCR30	Skin	5	8/213,944	4 ± 1	0.006
AG30	Liver	5	38/170,685	22 ± 7	
SCR30	Liver	5	13/296,783	4 ± 2	0.0004
AG30	Kidney	5	34/110,937	27 ± 13	
SCR30	Kidney	5	12/185,818	6 ± 2	0.007
AG30	Colon	2	29/76,930	38 ± 4	
SCR30	Colon	2	8/73,275	11 ± 0	
AG30	Intestine	2	8/18,214	46 ± 13	
SCR30	Intestine	2	4/46,397	9 ± 5	
AC30	Lung	2	13/56,370	23 ± 4	
SCR30	Lung	2	4/83,190	5 ± 1	

Table 2. Lack of TFO-induced mutagenesis of the *cII* gene in Mutamice. Mice were injected i.p. with 1 mg of oligonucleotide (either AG30 or SCR30) per day for 5 days (~50 mg kg^{-1} day^{-1}) or with PBS.

TFO	Tissue	No. of mice	Mutants/total plaques	Mutation frequency ($x10^{-5}$)
AG30	Skin	2	14/337,500	4
SCR30	Skin	2	19/344,500	6
None (PBS)	Skin	2	16/271,000	6

13. I declare that all statements made herein of my own knowledge and belief are true and that all statements made on information and belief are believed to be true, and further, that the statements are made with the knowledge that willful false statements are punishable by fine or imprisonment, or both, under section 1001 of Title 18 of the United States Code, and that such willful and false statements may jeopardize the validity of the application or any patent issuing thereon.

Date: 7/10/02

Peter M. Glazer, M.D., Ph.D.

ATL1 #533112 v1